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Benefits of Mixed-Mode Cation Exchange Chromatography: CMM HyperCel Resin Used for Monoclonal Antibody and Other Recombinant Protein Purification

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1. Summary

This note addresses the performance of mixed-mode cation exchange (CEX) CMM HyperCel resin, which can be applied to both monoclonal antibody (mAb) and recombinant protein purification. The study highlights the behavior of the CMM HyperCel resin versus conventional cation exchange (sulfopropyl groups) and a weak cation exchange multi-modal resin (cross-linked agarose) in terms of dynamic binding capacity (DBC) and selectivity (aggregate removal). Results demonstrate that compared to conventional cation exchangers, CMM HyperCel resin has a higher DBC (>60 mg/mL at pH 5.0, 25 mS/cm) over a broader range of pH (5.0 to 8.0) and conductivity (5 to 45 mS/cm). This capability provides maximal flexibility during process development, allowing streamlined operation with direct load of samples from previous process steps without feed adjustment. When used in a polishing sequence, the selectivity of CMM HyperCel resin allows good resolution of monomeric mAb from aggregates and removal of host cell proteins.

These unique properties can be of benefit for improved purification of engineered antibodies, antibody fragments, or various recombinant proteins with very close isoelectric points (pI) and | or degrees of hydrophobicity.

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2. Introduction

Mixed-mode or multi-modal chromatography offers an alternative to conventional purification methods such as ion exchange chromatography and has been increasingly used to purify proteins with closely related isoelectric points or hydrophobicities (engineered antibodies, fragments, charged variants or various recombinant proteins).

In a mAb purification platform, a unique mixed-mode cation exchanger, CMM HyperCel resin, can be used either as the second step after Protein A or as the third chromatography step, following mAb capture using Protein A , and anion exchange operated in FT mode step. In the latter case CMM HyperCel resin mainly contributes to host cell protein (HCP) and aggregate and remaining host cell DNA removal. In the following application,a viral inactivation step at acidic pH (3.6) was performed after the affinity step.

Both DBC and selectivity (in this case aggregate removal) are key parameters to achieve drug product critical quality attributes, and should therefore be carefully evaluated during process development, independent of unit operation order.

3. Materials and Methods

3.1. Materials

▪ Monoclonal Antibody Feedstocks

Adalimumab, Anti-Her2 (humanized IgG_1 monoclonal antibody that binds Her2), and Rituximab. mAb proteins were diluted to 2 g/L with the appropriate equilibration buffer. The pH was adjusted with 1 M acetic acid or 0.5 M Tris-Base. Conductivity was adjusted by addition of NaCl.

▪ Chromatography Resins

Resins for comparative studies are listed in Table 1. mAbs were loaded on 1 mL prepacked columns and all experiments were performed using an ÄKTA* avant 25 system (Cytiva).

Resins	Base Matrix	Ligand	Particle Size [µm]
CMM HyperCel resin	Highly cross-linked cellulose	Aminobenzoic acid	50-80
Multi-mode weak cation exchanger (MMAgarose)	Highly cross-linked agarose	Multimodal weak cation exchange	75
Sulfopropyl cation exchange (S-CEX) resin	Cross-linked polystyrenedivinylbenzene	Sulfopropyl ($-CH_2CH_2CH_2SO_3-$)	50

Table 1: Resins used for comparative studies

3.2. Determination of Dynamic Binding Capacity

DBC was determined by the needed load volume [mL] at a specific mAb concentration required to achieve 5% breakthrough [BT]. DBC at 5% BT was calculated as follows

$$\text{DBC at 5\% BT} = \frac{(V5\% - DV) \times CL}{CV}$$

Where: V5%= Volume at 5% BT [mL]
DV = Dead volume [mL]
CL = mAb concentration in load [mg/mL]
CV = Volume of the column [mL]

3.3. Quantification of HCP and Aggregate Analysis

- HCP quantification was conducted using CHO HCP ELISA kits, 3G (F550, Cygnus technologies).
- High performance liquid chromatography – size exclusion chromatography (HPLC–SEC) was chosen for soluble aggregate analysis (TSKgel* SuperSW3000 4.6 mm × 30 cm SEC column, Tosoh, P/N 18675) using a mobile phase of 20 mM sodium phosphate, pH 6.7, 0.5 M sodium perchlorate.
- Dip and Read Protein A (ProA) biosensors (Fortebio, P/N 18-5010) and the ForteBio Octet®* Red 96 biolayer interferometry (BLI) system were used to determine the mAb concentration of harvest cell culture fluid (HCCF) samples.
- A NanoDrop* 8000 spectrophotometer (Thermo Fisher Scientific) was used to determine the mAb concentration of purified mAb samples by UVA_{280}

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4. Experiments and Results

4.1. Comparison of Dynamic Binding Capacities

A broad range of loading conditions were explored, from pH 5.0 to 8.0 and conductivity of 5 to 45 mS/cm. Chromatography steps and buffers are shown in Table 2, and DBC histograms in Figure 1.

Step	Residence time [min]	Volume	Buffer
Equilibration	1	5 or 10 column volumes (CV)	25 mM Na-acetate pH 5.0 at 5, 25, or 45 mS/cm 25 mM Na-phosphate pH 6.5 at 5, 25, or 45 mS/cm 25 mM Tris-HCl pH 8.0 at 5 or 25 mS/cm
Load	4	To 5% BT	
Wash 1	1	2 or 5 CV	Equilibration buffer
Wash 2	1	10 CV	25 mM Na-phosphate pH 6.5, 25 mS/cm
Strip	1	5 CV	25 mM Tris-HCl pH 8.0, 45 mS/cm
Cleaning-in-place (CIP)	1	5 CV	1 M NaOH

Table 2: Chromatography method used for DBC study

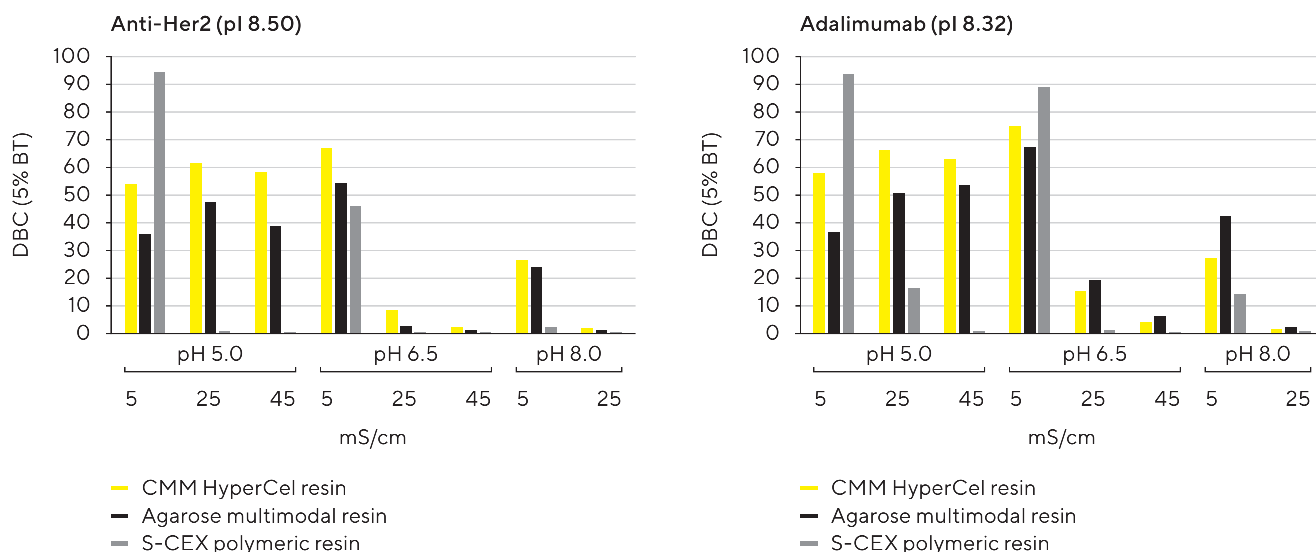


Figure 1: Comparative DBC study according to loading buffer pH and conductivity: DBC at 5% breakthrough (BT) for Anti-Her2 and Adalimumab using CMM HyperCel, agarose multimodal, and sulfopropyl polymeric cation exchange (S-CEX) resins

4.2. Results

As shown in Figure 1, the S-CEX resin exhibits higher DBC at low pH and conductivity (pH 5.0, 5 mS/cm). In contrast, CMM HyperCel resin – and to a lesser extent, the weak cation multi-mode agarose (MM-Agarose) resin – has a significantly higher DBC than S-CEX resin at higher conductivity (up to >60 mg/mL at pH 5.0, 45 mS/cm). This shows the power of, CMM HyperCel resin enabling operation over a broad range of pH (5.0 to 8.0) and conductivity (5 to 45 mS/cm), increasing process flexibility.

4.3. Comparison of Selectivities (Aggregate Removal Efficiency)

The selectivity performance of CMM HyperCel resin was compared to S-CEX and MM-Agarose resins. Selectivity is defined as aggregate removal with respect to product yield. Conductivity elution gradient analysis was used to characterize the selectivity properties of the three resins. Elution gradients at different constant pH values (6.5 and 7.5) are shown in Figures 2 and 3. The elution peaks were fractionated. Each sample was subjected to mAb concentration analysis (by UV) and mAb aggregate analysis (by size exclusion chromatography).This enables the determination of accumulated recovery of mAb through the elution peak as well as the associated % aggregate. As aggregates typically elute with higher conductivity than that of mAb monomers, this allows the identification of the % of product that can be recovered before a certain threshold level of aggregate is achieved.

For example, in Figure 2C, if the target aggregate level is below 0.5%, the resulting mAB yield achieved with S-CEX resin will be <50%, with MM-Agarose <90% and with CMM HyperCel >90%.

At constant pH, each process included a conductivity elution gradient (from 5 mS/cm to 100 mS/cm) spanning over 20 column volumes (CV). Runs were conducted with conductivity elution gradients at a constant pH of 6.5 (S-CEX resin) and pH 7.5 (Anti-Her2, CMM HyperCel and MM-Agarose resins). These pH values were selected as more appropriate for each type of resin. To increase resolution within the elution gradients, the columns were underloaded with a total of 10 mg of protein per 1 mL column with a residence time (RT) of 4 minutes.

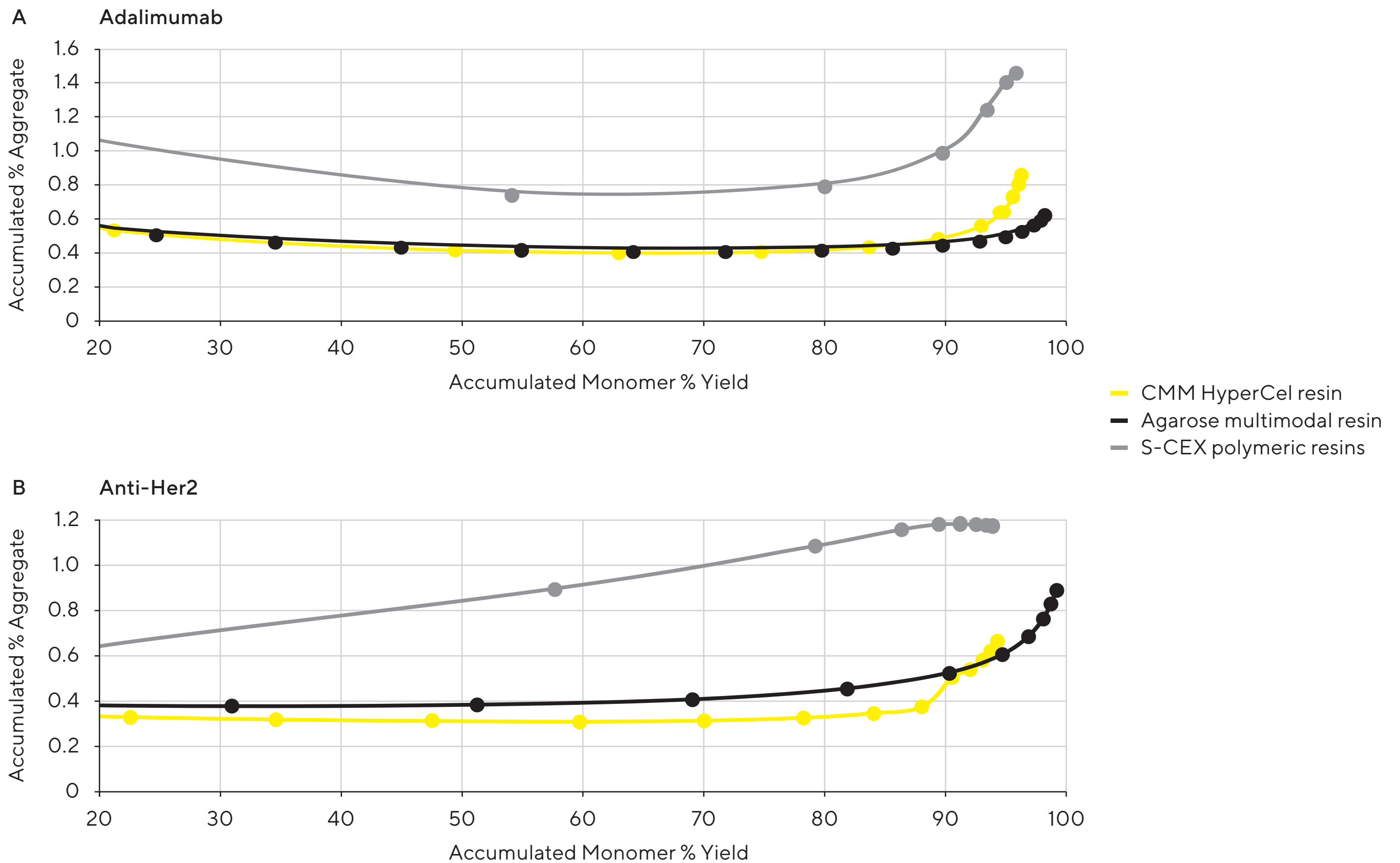


Figure 2: Conductivity elution at constant pH 6.5, 5 mS/cm to 100 mS/cm: Accumulated percent aggregate versus percent yield across the conductivity elution gradients.

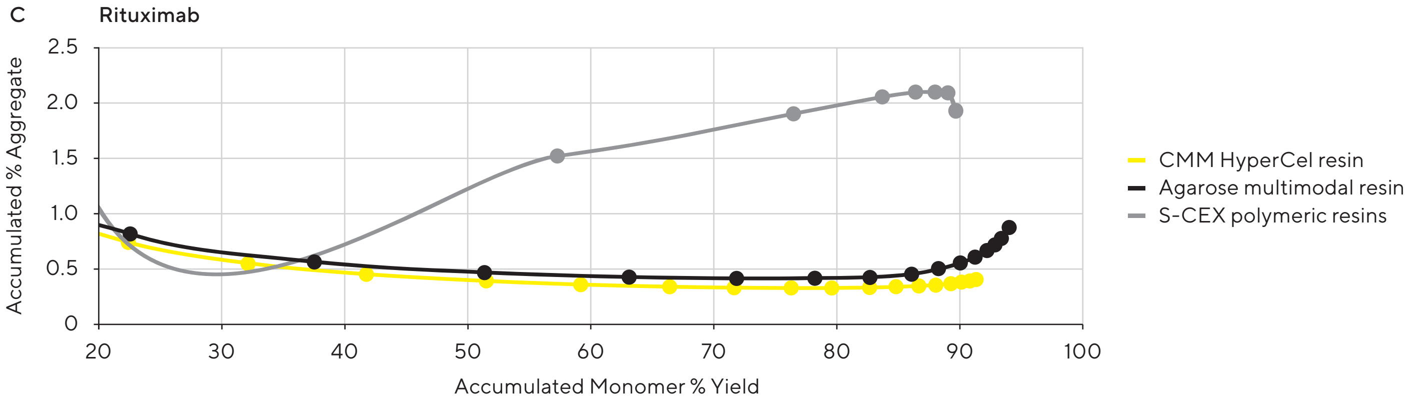
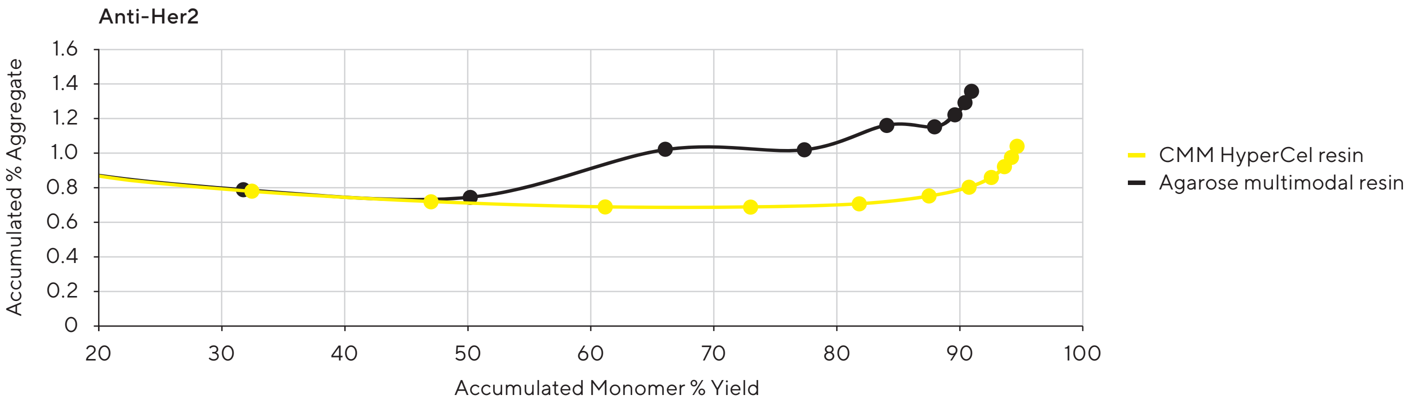


Figure 3: Conductivity elution gradients at a constant pH of 7.5, 5 mS/cm to 100 mS/cm: Accumulated percent aggregate versus percent yield across the conductivity elution gradients.



4.4. Selectivity Comparison Results

- With conductivity elution gradients at a constant pH of 6.5, an increased aggregate removal was achieved with CMM HyperCel and the MM-Agarose resins compared to the S-CEX resin, for three different mAbs (Figure 2).
- With conductivity elution gradients at a constant pH of 7.5, both CMM HyperCel and MM-Agarose resins showed superior aggregate removal compared to the S-CEX resin. However, CMM HyperCel resin outperformed the MM-Agarose resin (Figure 3).

Aggregate removal results are summarized in Table 3, showing the accumulated percent aggregate at 90% yield of mAb protein for each conductivity elution gradient.

mAb (initial aggregate % in the load)	Eluted: % Aggregate Protein vs.Monomer (at 90% mAb Recovery)			
	Gradient Elution	**S-CEX**	**CMM HyperCel**	**MM-Agarose**
Adalimumab(load: 1.2%)	pH 6.5, 5 mS/cm to 1 M NaCl	1.0	0.5	0.4
Rituximab (load: 2.0%)		2.0	0.4	0.6
Anti-Her2 (load: 1.0%)		1.2	0.5	0.5
Anti-Her2 (load: 1.0%)	pH 7.5, 5 mS/cm to 1 M NaCl	–	0.8	1.2
	pH 5.5, 5 mS/cm to 1 M NaCl	1.1	–	–

Table 3: Selectivity: comparative aggregate removal performance

5. More Flexibility and More Process Options

As shown in this study, the CMM HyperCel mixed-mode cation exchanger maintains a high DBC over a broad range of pH and conductivity. This feature allows for more flexible process development, enabling process streamlining and a optimal combination with previous purification steps with feed adjustment.

5.1. Option 1: High pH Load, Streamlined (An integrated process without buffer or pH adjustments)

This process comprises of a Protein A capture, virus inactivation, intermediate step on anion exchanger in FT mode, and CMM HyperCel resin for final polishing. In this case, one condition of pH 8.0, 5 mS/cm could be used to both maximize HCP removal by the AEX step, and still achieves acceptable DBC during the subsequent CMM HyperCel resin polishing step in bind and elute mode. Such an integration cannot be performed with a conventional S-CEX resin (i.e., with two mAbs at pH 8.0, 5 mS/cm, a DBC >26 mg/mL obtained with CMM HyperCel resin versus 2 or 15 mg/mL with S-CEX resin depending on the mAb). Under this binding condition CMM HyperCel resin enables a streamlined process with a direct load of anion exchanger eluate on to the CMM HyperCel resin saving any buffer exchange or dilution step. The CMM HyperCel yield remains higher than 98%.

Elution from CMM HyperCel resin can be performed with pH 6.8 and 26 mS/cm in case of Anti Her2 or pH 7 and 27 mS/cm in case of Adalimumab, respectively. These mild conditions will preserve the product integrity and stability. There will be no further aggregation of the mAbs due to acidic pH.

Overall, the described process resulted in a final product with <14 ppm HCP, <0.5% aggregate and yield of >98% on the CMM HyperCel resin. The final specifications of the mAbs are in line with regulatory expectations.

Using CMM HyperCel during the third step in a mAb process gives the opportunity to maintain the same pH, conductivity from the prior ion exchange step on the mixed mode resin. This saves a diafiltration step and labor time. A perfect match to intensify the process later on and convert it into a continuous mode leading to increased productivity.

In case a streamlined process is not an option, CMM HyperCel resin can be used in a more traditional way after Protein A bringing capacity and high selectivity compared to that of cation exchangers. The conditions of use will be optimized according to the mAb specificity only, without any compromise due to the technical constraint of the previous step.

5.2. Option 2: High Conductivity Load

This option can be used for other proteins than mAb, for example to process an eluted product from a preceding step within a multi-step purification. As an example, recombinant proteins, Fab fragments, engineered antibodies purification processes may include multiple bind and elute steps. Such chromatography steps may require high conductivity elution. For other products it could be necessary to keep a high conductivity to preserve protein stability. Such feedstocks with high conductivity may then be directly loaded onto CMM HyperCel columns without buffer exchange, pH adjustments or any dilution, which would not be possible with conventional ion exchange or hydrophobic interaction chromatography resins. At the same time, such a high conductivity load would not significantly jeopardize DBC, leading to improved process economics and throughput.

6. Conclusion

- CMM HyperCel resin has a higher DBC (e.g. >60 mg/mL at pH 5.0, 25 mS/cm) compared to most other mixed -mode resins. It can be used over a broad range of pH (5.0 to 8.0) and conductivity (5 to 45 mS/cm) leading to a high selectivity.
- CMM HyperCel´s unique features provide maximal flexibility during process development and allow streamlined operation with direct load of samples from previous process steps without feed adjustment.
- With CMM HyperCel enhanced process economics like saving unit operation costs such as ultrafiltration | diafiltration (UF | DF) and total buffers costs can be achieved.
- CMM HyperCel has superior selectivity compared to S-CEX and multi-mode resins, leading to improved mAb aggregate removal. Which is in line with regulatory expectations for mAb product.
- The bespoke benefits are not limited to mAbs but can also contribute to an efficient purification of any recombinant proteins I engineered antibodies and antibody fragments.

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